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Easy Strategy To Protect Antimicrobial Peptides from Fast Degradation in Serum▽

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Antimicrobial peptides are promising novel peptide leads, but their low serum stability often limits their further consideration in drug development programs. Here, we describe a generally applicable strategy to stabilize peptides against serum proteases by replacing arginine residues with α-amino-3-guanidino-propionic acid (Agp). Peptide NH_2-RRWRIVVIRVRR-$CONH_2$ was nearby totally degraded after 8 h in mouse serum, whereas the variant with Agp substituted was degraded less than 20%. The antimicrobial activity was not affected.

Many infectious diseases are a big threat to humankind and could kill a huge proportion of the population. For example, the last plague pandemic in central Asia killed around 12 million people. The introduction of antibiotics onto the market gave medicine a new and effective tool to treat bacterial infections. Based on that success, novel techniques in modern Western medicine could be developed to the extent we know today. With the rise of multidrug-resistant bacteria, it could be possible that bacterial infections will become again a new threat to Western medicine. Therefore, there is a great urgency to find novel substances/strategies to fight against multidrug-resistant bacteria. One class of substances that have been shown to be active against multidrug-resistant bacteria are antimicrobial peptides, also called host defense peptides. These peptides stem from the innate immune system. For treatment of catheter infections and rosacea, the peptide omiganan has been undergoing phase III clinical studies for both indications (1, 4). However, the fast development of peptide-based drugs is often hampered by the fact that the peptides will be degraded by proteases in the serum, especially antimicrobial peptides that are cationic and show fast degradation due to their arginine and lysine content. Here we show an easy strategy that makes use of an amino acid derivate of arginine, 9-fluorenylmethoxy carbonyl (Fmoc)-L-Agp$(Boc)_2$-OH (Agp [α-amino-3-guanidino-propionic acid]), which is suitable for standard Fmoc peptide synthesis. Agp is commercially available (Iris Biotech GmbH) and is about 20-fold more expensive than Fmoc-protected Arg. It has already been shown that the Agp could dramatically reduce the cleavage of a model peptide by trypsin (2).

First, an arginine-rich short antimicrobial peptide was selected from previous investigations (peptide Sub3; NH_2-RRW RIVVIRVRR-$CONH_2$) (3), and this peptide and the analog consisting of Agp instead of Arg (Sub3-Agp; NH_2-AgpAgpW AgpIVVIAgpVAgpAgp-$CONH_2$) were synthesized by Fmoc chemistry. In addition, both of these peptides were also synthesized having D-Ala (a) and D-His (h) added to the N and C termini to prevent cleavage by exoproteases, and resulting in Sub3-D (NH_2-aRRWRIVVIRVRRh-$CONH_2$) and Sub3-D-Agp (NH_2-aAgpAgpWAgpIVVIAgpVAgpAgph-$CONH_2$).

The stepwise solid-phase synthesis of the peptide amide Sub3 and Sub3-D was performed on an automated ABI 433A peptide synthesizer from Applied Biosystems on a 0.07 mM scale, using conventional Fmoc/tBu strategy. Sieber resin (350 mg, loading at 0.2 mmol/g) from Novabiochem was used as solid support. All amino acids (1 mmol) were coupled with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) activation according to standard methodologies in an automated single- or double-coupling mode. Fmoc deprotection was carried out with 20% piperidine in *N*-methyl-2-pyrrolidone. The peptides Sub3-Agp and Sub3-D-Agp were synthesized manually, using a 350-mg Sieber resin, 0.2-mmol/g scale. After each coupling, a Kaiser test was made. Cleavage of crude peptides from their resins was accomplished through treatment with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane for 3.5 h. Preparative purification by high-pressure liquid chromatography (HPLC) was carried out on a Shimadzu LC-8A system with a Zorbax C_{18} column (21.2-mm internal diameter, 250-mm length, 7-μm particle size) with eluent A (0.2% TFA in water) and eluent B (0.2% TFA in 1:4 water-acetonitrile). The peptides were purified with a gradient of 29 to 54% eluent B in 50 min.

The effect of these homologues on the antimicrobial activity against wild-type *Pseudomonas aeruginosa* PAO1 strain H103 and *Staphylococcus aureus* ATCC 25923 was tested. The MIC of the peptides was measured by using a modified broth microdilution method in Mueller-Hinton (MH) medium (5). The peptides were dissolved in distilled water and stored in glass vials. The assay was performed in sterile 96-well polypropylene microtiter plates (Greiner). Serial dilutions of the peptides to

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TABLE 1. Sequences and MIC values of Sub3 and its analogs

Peptide name	Peptide sequence[a]	MIC (μg/ml)[b]	
		S. aureus	*P. aeruginosa*
Sub3	RRWRIVVIRVRR-$CONH_2$	1	2
Sub3-Agp	AgpAgpWAgpIVVIAgpVAgpAgp-$CONH_2$	1	2
Sub3-D	aRRWRIVVIRVRRh-$CONH_2$	1	2
Sub3-D-Agp	aAgpAgpWAgpIVVIAgpVAgpAgph-$CONH_2$	1	2

[a] a denotes D-Ala and h denotes D-His.
[b] Antibacterial activities were determined against *Staphylococcus aureus* and *Pseudomonas aeruginosa* using a modified broth microdilution method in Mueller-Hinton medium.

be assayed were performed in 0.01% aqueous acetic acid (Fisher) containing 0.2% (wt/vol) bovine serum albumin (Boehringer Mannheim GmbH) at 10× the desired final concentration. Bacteria were added to the plate from an overnight culture at 2×10^5 to 7×10^5 CFU/ml and incubated overnight at 37°C. The MIC was taken as the concentration at which no growth was observed. The results of the test are given in Table 1. All peptides show the same antimicrobial activity. Even though the guanidino group of the Agp is by two carbon bond lengths closer to the backbone than that of arginine, the MIC is not changed. Surprisingly, the distance of the positive charge of the antimicrobial peptides from the negatively charged lipid heads of the bacterial membrane seems not to play a role in the antibacterial activity. In a study using α-helical cell lytic peptides, the exchange of lysine against ornithine and 2,4-diaminobutyric acid (Dab), also had only a small effect on the MIC; however, faster kinetics was seen, due to the snorkel effect (6).

The serum stability of all peptides was determined in 25% (vol/vol) aqueous pooled mouse serum (PAA Laboratories GmbH, Pasching, Austria). Peptides were dissolved in serum at a final concentration of 150 μg/ml and incubated at 37°C. Aliquots of 95 μl taken in duplicates after 0, 60, 120, 240, and 480 min were precipitated with a mixture of acetonitrile, water, and formic acid (300 μl; 89:10:1 by volume). After 45 min on ice, the samples were centrifuged (10 min, 12,000 × *g*, at 4°C), and the supernatants were dried under vacuum and stored at −20°C. The samples were analyzed on a Jupiter C_{18} column (internal diameter, 4.6 mm; length, 150 mm; particle size, 5 μm; pore size, 30 nm; Phenomenex, Inc., Torrance, CA) using a linear aqueous acetonitrile gradient containing 0.1% (vol/vol) TFA. Metabolites were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) (4700 proteomic analyzer; Applied Biosystems GmbH, Darmstadt, Germany) using α-cyano-4-hydroxycinnaminic acid (Bruker Daltonik GmbH, Bremen, Germany) as a matrix (4 mg/ml in 60% acetonitrile containing 0.1% aqueous TFA).

Replacement of Arg residues with Agp stabilized peptide Sub3 remarkably. As described earlier by Henklein et al. (2), the replacement of arginine by α-amino-3-guanidinopropionic acid (Agp) stabilizes specific protease cleavage sites. With a half-life of approximately 1 h in 25% aqueous mouse serum, Sub3 was relatively stable for a medium-sized peptide, consid-

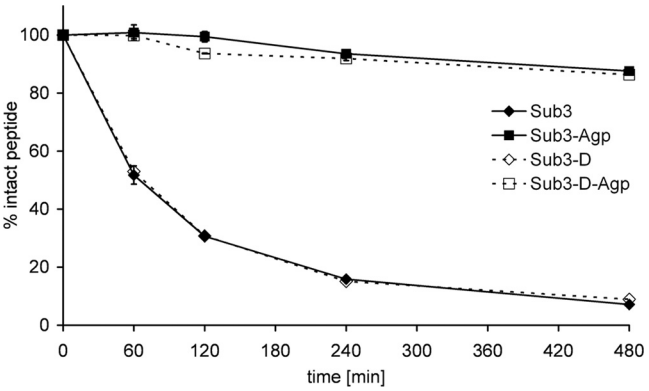

FIG. 1. Stability of Sub3 analogs in 25% aqueous mouse serum. Peptide amounts of Sub3 (solid line with solid diamonds), Sub3-Agp (solid line with solid squares), Sub3-D (dotted line with open diamonds), and Sub3-D-Agp (dotted line with open squares) were determined by the peak areas of the RP-HPLC relative to the peak areas obtained at t_0 (control at 0 min set to 100% for each peak).

ering that it does not adopt a stable structure and contains several tryptic cleavage sites (Fig. 1). Serum incubation yielded two additional peaks by reversed-phase (RP)-HPLC that were not present in the control (0 min) and thus most likely represented peptide degradation products (metabolites) that were further characterized by MALDI MS. The first peak, eluting approximately 1 min later than Sub3, showed two signals at *m*/*z* 1,507.96 and 1,508.94, indicating mass losses of 156 and 155 u compared to the parent peptide. These mass losses indicated cleavage of the N-terminal arginine residue or the C-terminal arginine amide residue, respectively, yielding Sub3(2-12) and Sub3(1-11) (Fig. 2). The second peak appearing in the chromatogram at a retention time of 1 h was detected at *m*/*z* 1,352.85, indicating simultaneous cleavage of the N- and C-terminal Arg residues [Sub3(2-11)] (data not shown). Elongation of the peptide sequence with D-alanine at the N terminus and D-histidine at the C terminus did not affect the peptide stability. This observation likely confirms that both terminal residues were cleaved by endoproteases and not exoproteases.

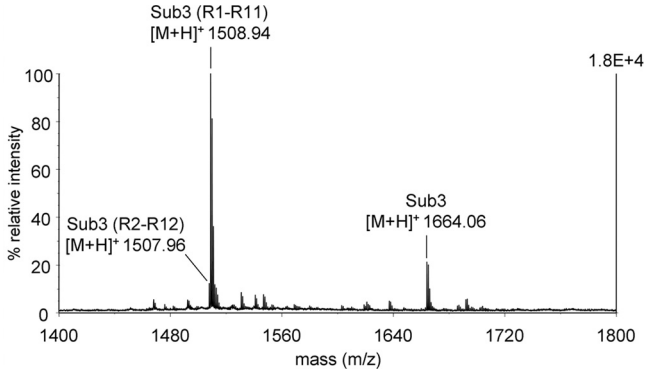

FIG. 2. MALDI mass spectrum of an RP chromatography fraction containing Sub3 and its two major degradation products Sub3(1–11) and Sub3(2–12). Sub3 was incubated in 25% aqueous mouse serum for 60 min and separated on the Jupiter C_{18} column before the main fraction was analyzed by MALDI-TOF MS. R1-R11 and R2-12, arginine residues 1 to 11 and 2 to 12, respectively.

In an attempt to reduce the proteolytic cleavage of Sub3 by serum proteases, all of the Arg residues were replaced with Agp (Sub3-Agp). Following this modification, the peptide stability was increased dramatically, with only 20% being degraded after 8 h incubation time. The stability was again not affected by elongating the sequence at the N and C terminus with D-Ala and D-His, respectively.

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